

Tissue interaction between the retinal pigment epithelium and the choroid triggers retinal regeneration of the newt *Cynops pyrrhogaster*

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Abstract

Complete retinal regeneration in adult animals occurs only in certain urodele amphibians, in which the retinal pigmented epithelial cells (RPE) undergo transdifferentiation to produce all cell types constituting the neural retina. A similar mechanism also appears to be involved in retinal regeneration in the embryonic stage of some other species, but the nature of this mechanism has not yet been elucidated. The organ culture model of retinal regeneration is a useful experimental system and we previously reported RPE transdifferentiation of the newt under this condition. Here, we show that cultured RPE cells proliferate and differentiate into neurons when cultured with the choroid attached to the RPE, but they did not exhibit any morphological changes when cultured alone following removal of the choroid. This finding indicates that the tissue interactions between the RPE and the choroid are essential for the former to proliferate. This tissue interaction appears to be mediated by diffusible factors, because the choroid could affect RPE cells even when the two tissues were separated by a membrane filter. RPE transdifferentiation under the organotypic culture condition was abolished by a MEK (ERK kinase) inhibitor, U0126, but was partially suppressed by an FGF receptor inhibitor, SU5402, suggesting that FGF signaling pathway has a central role in the transdifferentiation. While IGF-1 alone had no effect on isolated RPE, combination of FGF-2 and IGF-1 stimulated RPE cell transdifferentiation similar to the results obtained in organ-cultured RPE and choroid. RT-PCR revealed that gene expression of both FGF-2 and IGF-1 is up-regulated following removal of the retina. Thus, we show for the first time that the choroid plays an essential role in newt retinal regeneration, opening a new avenue for understanding the molecular mechanisms underlying retinal regeneration.

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Keywords: Newt retina; Regeneration; RPE; Choroid; Transdifferentiation; FGF-2; IGF-1

Introduction

Complete retinal regeneration in adult animals occurs only in certain urodele amphibians following surgical removal of the retinal tissue. This has been best studied in the newt over a century since the time when experimental studies of the phenomenon were initiated at the end of 19th century (see reviews by Mitashov, 1997; Okada, 1991), although we still have very little information on the cellular and molecular mechanisms involved. Cells in the retinal pigmented epithelium (RPE) undergo

transdifferentiation to produce all cell types constituting the neural retina. In the adult newt, the retina regenerates both from the ciliary epithelium and the RPE, the latter being the major source of the regenerating tissues. The regeneration processes have been fully documented in previous papers (Hasegawa, 1958; Reyner, 1971, 1977; Stone, 1950a,b). A similar retinal regeneration from the RPE cells also occurs in some other species including birds and mammals, but in which the regenerative capacity is strictly limited to early stages of embryonic development (Araki et al., 1998; Park and Hollenberg, 1989; Zhao et al., 1995). Avian and mammalian RPE cells require extrinsically administered factors such as fibroblast growth factors (FGFs) for the onset of transdifferentiation, and the transdifferentiated RPE can no

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longer reform a new RPE (Del Rio-Tsonis and Tsonis, 2003).

Recent cell culture studies have revealed that retinal stem cells sequester in the ciliary marginal body of the adult mammalian eye (Haruta et al., 2001; Tropepe et al., 2000), and several growth factors like FGFs appear to regulate their growth and cell differentiation. It was also shown in the newly hatched chick retina that glial cells retain the capacity for neuron differentiation by treating the eyes with growth factors in vitro and in oculo (Fischer et al., 2002). Thus, the understanding of newt retinal regeneration has strategic importance for further insight into vertebrate retinal regeneration. Regeneration of the retina in the urodele amphibian is highly reproducible and does not require addition of exogenous factors, indicating that this system possesses all the necessary elements and that understanding the regulatory mechanisms involved will provide valuable clues to retinal regeneration in other species. The most important questions are: which cellular and molecular mechanisms are involved in newt retinal regeneration, and what endogenous factor(s) is/are involved in triggering this process? And why can the newt retina regenerate even at the adult stage?

We previously reported newt RPE transdifferentiation using a novel organ culture model (Ikegami et al., 2002) and here we extend this model to investigate the role of ocular tissue interactions in newt retinal regeneration. We show that cultured RPE cells proliferate and differentiate into neurons only when cultured with the adjacent choroid, indicating that specific tissue interactions are essential for RPE proliferation and that RPE transdifferentiation does not require retina-derived factors. We also show that this interaction is mediated by several diffusible factors, FGF-2 and IGF-1 (or insulin) being among the candidate molecules. Hence, it is shown for the first time that the choroid plays an essential role in newt retinal regeneration, opening a novel pathway for understanding the mechanisms controlling newt retinal regeneration.

Materials and methods

Animals

Adult female newts, *Cynops pyrrhogaster*, were purchased from a local supplier (Hamamatsu Seibutsu Kyouzai Ltd., Shizuoka, Japan). The newts were kept in tap water at $20 \pm 2^\circ\text{C}$, and fed with mixed feed, originally prepared for fish culture (Taiyou Siryou).

Tissue culture

The procedure for tissue culture was basically the same as described previously (Ikegami et al., 2002). The newts were deeply anesthetized with 0.15% MS222 (SIGMA), then decapitated and the heads immersed twice in 70% ethanol for

sterilization, each for 30 s, followed by washing with newt-Hanks' balanced salt solution (Okamoto et al., 1998). The eyeballs were enucleated carefully, and adherent muscles and fat bodies were removed cleanly. The anterior parts of the eyeballs including the irido–corneal complex and lens were discarded, and the posterior eyecups were kept in Ca^{2+} , Mg^{2+} -free newt-Hanks' solution. This treatment promoted ready detachment of neural retina from the RPE. The sclera was then removed and the remaining tissues consisting of the RPE and the choroidal membrane were placed flat on a filter cup membrane (Millicell-CM, pore size $0.4 \mu\text{m}$, Millipore), the choroid facing the filter membrane. The membrane was pre-coated with type I collagen (Cellmatrix TypeI-C, Nitta Gelatin). Each filter cup was placed in one well of a 6-well culture plate (#3046, Falcon). The medium was Livowitz L15 (GIBCO BRL) (diluted to 66% of the prescribed concentration for mammalian cell culture) supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories Inc.) and kanamycin sulfate (8 mg/dl, SIGMA). Cultures were maintained in a humidified dark incubator at 25°C and were fed with a fresh medium every 5 days. Growth factors, such as FGF-2 (Boehringer Mannheim Biochemica), IGF-1 (Gro-Pep), insulin (SIGMA), EGF (Becton Dickinson), CNTF (SIGMA) and PDGF (Austral Biologicals) were added to the culture medium together with $7.5 \mu\text{g/ml}$ heparin sulfate (Wako Pure Chem.) 24 h after the culture was initiated.

To obtain a single RPE sheet without any connective tissue, the RPE was detached from the choroid after the tissue was incubated in Dispase solution (50 unit/ml, Godo Shusei Ltd., Japan) for 40 h at 25°C . Usually, a whole RPE sheet from each eye was obtained with minimum damage to the peripheral region. Histological preparation for the isolated RPE sheet confirmed that the choroid had been removed cleanly.

To inhibit the FGF signaling pathway, cultures were treated with SU5402 (CALBIOCHEM) or U0126 (PROMEGA). SU5402 binds specifically to the active sites of FGFR kinase domains (Mohammadi et al., 1998) and U0126, a MEK inhibitor, inhibits both the activation of MEK by Raf and the activation of ERK by MEK (Favata et al., 1998). SU5402 and U0126 were dissolved in dimethylsulfoxide (DMSO) and added to the medium every 3 days.

Ryomen culture of the choroid and RPE sheet

The RPE sheets were co-cultured with individual choroids by separating these two tissues by a membrane filter according to the method described by Ichijo and Bonhoeffer (1998). The isolated choroids were placed with the basal surface on the membrane filter (Falcon Cell Culture Insert) and covered with collagen gel. Two or three days later, freshly isolated RPE sheets were positioned basal surface downwards on the opposite side of the filter, reconstituting the correct polarity observed in vivo. They were further cultured for 30–35 days.

Reverse transcriptase (RT)-PCR analysis of FGF-2 and IGF-1 gene expression in the retina and choroid

RNA was extracted from the normal newt retina and choroid. 1 µg of total RNA was reverse-transcribed in 30 µl of reaction mixture containing the RT buffer, 1 µg of oligo(dT), 0.1 mM of each deoxynucleotide triphosphate (dNTP), 10 U of RNase inhibitor and 300 U of Molony murine leukemia virus reverse transcriptase. PCR was performed in the final volume of 50 µl of PCR reaction mixture (Toyobo Co., Ltd.) containing 0.1 mM of each dNTP, 50 pmol of each primer and 2.5 U of Taq polymerase. The amount of cDNA added to the reaction mixture was normalized by the intensity of glucose 3-phosphate dehydrogenase amplicon. Aliquots were analyzed by 1.1% agarose gel. The primers used were newt FGF-2 upstream primer (5' to 3') (gctgtactgcaagaacg), newt FGF-2 downstream primer (5' to 3') (gccacataccaatcgga), newt IGF-1 upstream primer (aaggtgaagatgcacacc), newt IGF-1 downstream primer (ccctcggtgactgtgccgt), newt EF1-α upstream primer (gaccttggccccagtaacgtaaccac) and newt EF1-α downstream primer (actgggtgttctgctggcgtactcttg). The level of RNA was semi-quantified densitometrically.

Histological preparation

For histological observations, cultured tissues were fixed with a Bouin solution and embedded in paraffin. Sections of 6 µm thickness were cut in a plane perpendicularly to the membrane filter and stained with either Azocarmine G/Aniline Blue or Hematoxylin/eosin.

Immunocytochemistry of cultured cells

Cultures were fixed with an ice-chilled mixture of 2% paraformaldehyde and 0.5% glutaraldehyde in 50 mM phosphate-buffered saline (PBS, pH 7.4) for 10 min, followed by a second fixation for 3 h with 2% paraformaldehyde in PBS. After washing with PBS, they were processed for immunocytochemistry with fluorescence-labeled secondary antibodies. First, they were treated for 1 h with PBS containing 2% FBS and 0.2% Triton X-100, and then incubated in a primary antibody for 2 h at room temperature. They were washed three times each for 5 min with PBS

containing 0.2% Triton X-100 and then twice for 5 min with PBS, followed by an incubation with the secondary antibody for 1 h at room temperature. The secondary antibodies used were anti-rabbit and anti-mouse IgG conjugated with either Alexa-488 or Alexa-594 Fluorescent (Molecular Probes). The primary antibodies used were anti-HPC-1 (Syntaxin) (Akagawa et al., 1990), anti-bovine rhodopsin (1D4) (Mol-day and Mackenzie, 1983), anti-acetylated tubulin (SIGMA; Piperno and Fuller, 1985), anti-neurofilament 200 (SIGMA) and anti-BrdU (SIGMA; Gratzner, 1982). They were diluted at 1:1000, 1:300, 1:1000 and 1:500, respectively. The specificity of these antibodies has been described in previous papers.

BrdU labeling

To detect proliferating cells under the culture conditions, BrdU (5-bromo-2'-deoxyuridine; SIGMA) was administered to the medium at 5 µg/ml. Cultures were fixed as described above and treated with 2 N HCl for 1 h before being subjected to immunocytochemistry.

Intraocular transplantation of cultured tissues

To know whether cells transdifferentiating from RPE can form a stratified structure resembling the neural retina, tissues cultured in vitro for 10 days were detached from the filter membrane and inserted into the anterior chamber of a lenticotomized newt eye, the RPE surface always facing the vitreous chamber. In the case of a single RPE sheet, cultured RPE sheets attached on the filter membrane were inserted together into the ocular chamber. After a certain period, the eyes were enucleated and fixed with a Bouin solution for histological observations.

Results

Organotypic cultures of the RPE–choroid and the isolated RPE sheet

For organotypic culture of RPE–choroid tissues, the sclera was removed from the posterior regions of retinec-

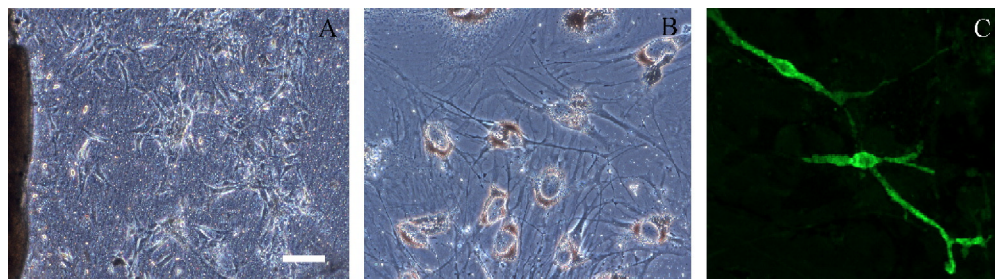


Fig. 1. Outgrowth of RPE cells and neuronal differentiation. (A,B) Phase-contrast micrographs of cells migrating out from an explant (RPE and choroid) located at the left side (asterisk). Cells extended long branching neurite-like processes, and many of them still contained pigment granules. Day 20. (C) Syntaxin1A-immunoreactive cells were found among them. Scale bar is 250 µm for panel A, 50 µm for panel B and 100 µm for C.

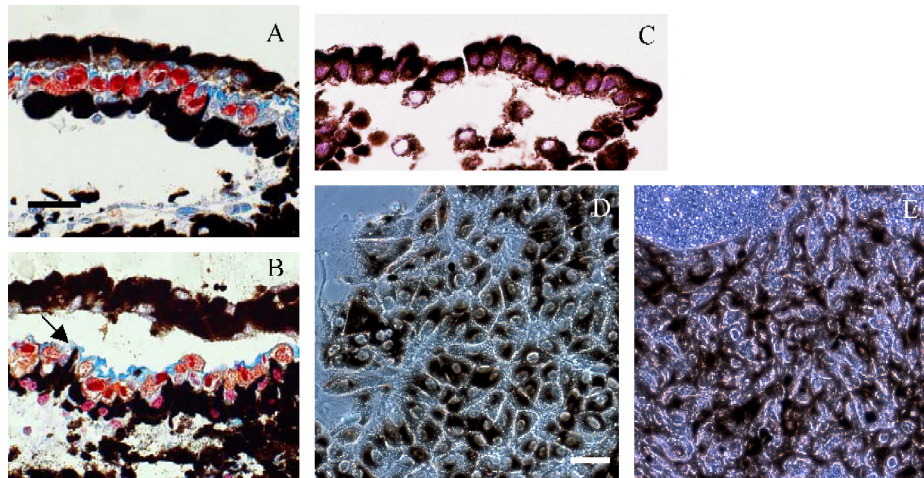


Fig. 2. Isolation of RPE sheet from the choroid and culturing of isolated RPE. (A) A tissue consisting of RPE and choroid (sclera had been removed) stained for Azan. (B) Dispace treatment removed RPE from the choroid. The Bruch's membrane stained in blue color is found at the choroid (arrow). (C) Isolated RPE sheet. No other cells from the choroid were found. (D) Isolated RPE sheet cultured on the membrane for 2 days showed an epithelial feature which remained on day 30 as seen in panel E. Scale bar in panel A is 100 μ m and is applied to panels B and C. Scale bar in panel D is 100 μ m and is applied to panel E.

tomized eyes and the remaining RPE with choroid was cultured on the membrane filter. Numerous RPE cells migrated out from the epithelial sheet from about days 3 to 5 *in vitro* and gradually lost pigmentation. Commencing about days 7 to 10, they showed a neuron-like morphology with a few long branching neuritic processes as observed in previous studies (Ikegami et al., 2002) (Fig. 1). Immunocytochemical detection of various neuron-specific markers revealed that they were positively stained for HPC-1 (Syntaxin1A), neurofilament, acetylated tubulin or rhodopsin. Administration of FGF-2 in the culture media promoted considerably neural cell differentiation.

By treating RPE–choroid tissues with dispace, a single RPE layer was isolated (Figs. 2A–C). Histological preparation revealed that RPE layers were separated at the level of the basement membrane (Bruch's membrane), which remained attached to the choroid (Fig. 2B). Connective tissue cells had been cleanly removed from the RPE layers. RPE cells in the isolated RPE layer were tightly attached to each other, suggesting that dispace treatment did not affect the cell–cell adhesion (Fig. 2C). Isolated RPE sheets were cultured on the filter membrane, the basal side facing the filter membrane. Within a few days, RPE cells were firmly attached to the membrane and formed a simple densely pigmented epithelial layer (Fig. 2D). These features were essentially the same on day 30, the epithelial structure of the RPE being well maintained (Fig. 2E). To examine whether cells in the RPE sheet proliferated or not, cultures were labeled with BrdU for 20 days (from Days 1 to 20) and were stained immunocytochemically for BrdU. Very few nuclei were positively stained, indicating that the vast majority of cells did not undergo cell division (Fig. 4B).

To exclude the possibility that cells of the isolated RPE sheets had lost proliferative activity due to cytological damage by dispace treatment, an isolated RPE sheet was subsequently reapposed to choroids attached to membrane

filters. The results showed that RPE cells migrated, proliferated and differentiated into neural cells, indicating that dispace-treated RPE cells had not lost the ability to grow and differentiate after enzyme treatment.

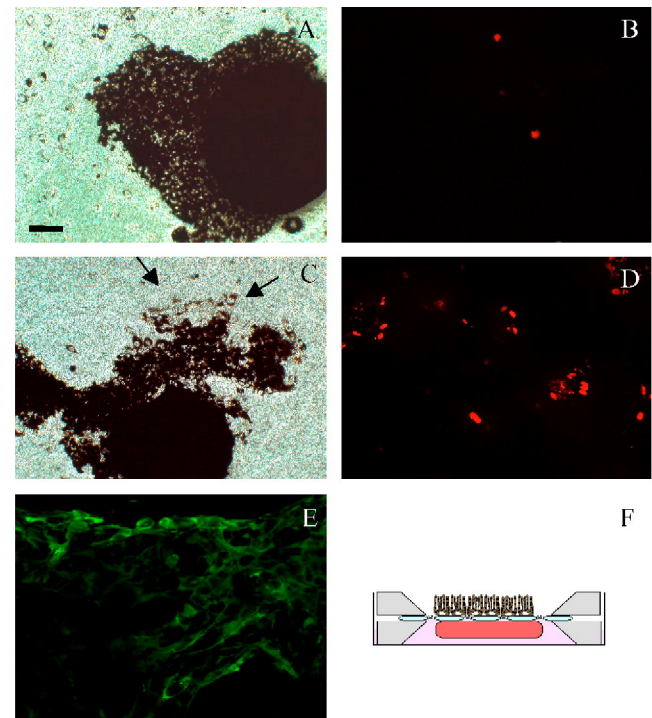


Fig. 3. Ryomen culture of RPE sheet and the choroid. These two tissues were cultured with a membrane filter separating them. RPE sheet was cultured either alone without the choroid (A, B) or with the choroid (C, D, E). In panel C, some of RPE cells migrated outward and became depigmented (arrows) and had proliferated as shown by the BrdU labeling (D). In panel C, numerous acetylated tubulin-immunoreactive cells were found. (F) A diagram of ryomen culture illustrating RPE sheet at the upper part and the choroid in the lower part. Scale bar in panel A is 200 μ m and is applied to panels B, C, D and E.

Ryomen culture of the RPE and choroid

These observations suggested that the choroid plays an important role in RPE cell transdifferentiation. To determine whether this interaction was contact-dependent or mediated by soluble factors, we utilized the ryomen culture method (Ichijo and Bonhoeffer, 1998); an isolated RPE layer was co-cultured with choroid, separated by a membrane filter. The results showed that RPE cells migrated out and became depigmented similar to the joined RPE–choroid explants (Fig. 3). BrdU-labeling experiments clearly showed that numerous RPE cells were labeled, whereas RPE cells cultured alone were rarely labeled with BrdU (Figs. 3B, D). Some of these cells extended long branching neuritic processes and were positively stained for acetylated tubulin (Fig. 3E). These results suggest that some diffusible factors are involved in RPE–choroid interaction.

Effect of growth factors on RPE cell growth and differentiation

To identify candidate molecules, growth factors were examined for their effects upon proliferation and/or neural differentiation using isolated RPE sheets. EGF, IGF-1, PDGF, CNTF and insulin showed no significant effect on RPE cells; the RPE sheet retained its epithelial features and remained densely pigmented. When cultured in the presence of BrdU for 20 days, very few nuclei were stained for BrdU, similar to the RPE cells cultured without growth factors (data not shown). In contrast, FGF-2 had a drastic effect on RPE cells and exerted a substantial change in their morphology (Fig. 4A). Cells at the periphery of the sheet migrated outwards and gradually lost melanin granules. At about day 20, most of these cells had long branching

neurite-like processes. Immunocytochemical staining of the culture showed that numerous cells were stained either for acetylated tubulin or neurofilament protein (Figs. 4D–F). A smaller number of cells were stained for HPC-1 (Syntaxin1A) or rhodopsin. The epithelial sheet also appeared to grow in size, and mitotic figures were often observed in the sheets. BrdU labeling indicated that numerous cells in the sheet were labeled, particularly at the periphery of the sheet (Fig. 4C).

To examine whether there is a critical window of FGF-2 activity, we administered FGF-2 for different periods of culture. When FGF-2 was present only for the initial 5 days, the RPE cells did not show any significant change, whereas they became depigmented and migrated out from the sheet when FGF-2 was present for the initial 10 days. In the latter case, however, cells with a typical neuronal morphology were not found. In another experiment, FGF-2 was administered to the medium on day 6 and thereafter, and in these conditions RPE cells showed morphological changes similar to those observed when FGF-2 was present for the whole culture period. However, delay of FGF-2 addition until day 10 resulted in RPE cells retaining epithelial features and failing to redifferentiate into neural cells. This shows that by day 10 RPE cells have undergone substantial changes in their cellular properties and have become unresponsive to FGF-2. These results are summarized in Fig. 5.

Effects of FGF signaling pathway inhibitors on RPE cell growth and differentiation

To examine whether endogenous FGF-2 is involved in RPE transdifferentiation in co-cultured RPE–choroid, FGF signaling pathway inhibitors, SU5402 and U0126, were

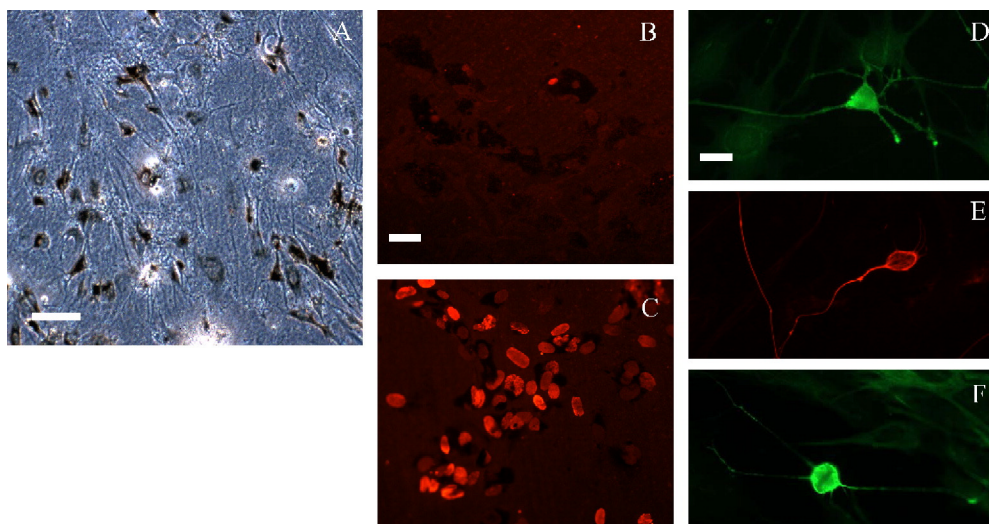


Fig. 4. Isolated RPE sheets were cultured with FGF-2 and examined for cell proliferation and neural differentiation. Numerous cells extended long neurite-like processes (A) and some of them were stained either for Syntaxin1A (D), acetylated tubulin (E) or neurofilament 200 kD (F). While RPE cells cultured without FGF-2 did not label for BrdU (B), numerous cells were labeled for BrdU when cultured with FGF-2, indicating they were proliferating in the culture (C). Scale bar in panel A is 100 μ m. Bar is 250 μ m for panel B and 50 μ m for panel C. Bar in panel D is 50 μ m and is applied to panels E and F.

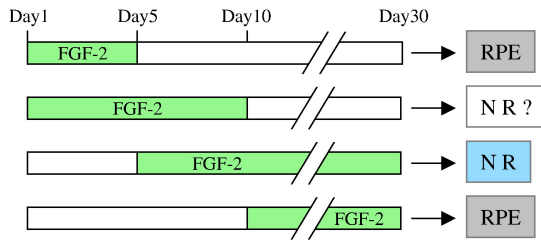


Fig. 5. Effect of FGF-2 administration period on RPE transdifferentiation. Purely isolated RPE sheets were cultured and were given FGF-2 for the duration indicated. Cultures were examined at day 30 whether they remained undifferentiated as pigmented epithelial form (RPE) or differentiated to retinal neurons (NR). When FGF-2 was present for the initial 10 days, RPE cells became depigmented, proliferated and migrated out, but they did not show neuronal features (NR?) (the second row).

added to the organotypic cultures. SU5402 and U0126 were administered at concentrations of 0.5, 1, 10, 20 and 50 μM , since they are reportedly effective between 10 and 20 μM for newt tissues (Hayashi et al., 2002; Zheng et al., 2000).

Cultures treated with 1 and 10 μM SU5402 did not differ from untreated cultures (without inhibitor, DMSO alone). When RPE–choroid tissues were treated with as much as 50 μM SU5402, the emergence of neuron-like cells from the RPE sheet was retarded by 3–5 days with respect to untreated cultures. BrdU-labeled cell numbers decreased slightly in cultures with 50 μM SU5402, whereas 10 μM SU5402 did not cause significant decrease in labeled cell numbers (Fig. 6A). The effect of SU5402 on neural differentiation was examined by HPC-1 (Syntaxin1A) and acetylated tubulin immunocytochemistry by counting positive cell numbers. The HPC-1-immunoreactive cell number was decreased substantially by the presence of 1 μM SU5402 (Fig. 6B), while the acetylated tubulin-immunoreactive cell number was affected only slightly by 50 μM (Figs. 6C, D). In contrast to the inhibitory effect by SU5402, the MEK

inhibitor, U0126, totally blocked RPE transdifferentiation at 20 μM (Fig. 7); RPE cells did neither proliferated nor migrated out from the epithelial sheet and only a small number of melanocytes migrated out when observed on day 25 (Fig. 7D). This inhibitory effect by U0126 was reversible and RPE cells migrated out, became elongated and finally differentiated into neurons when cultures were free of the inhibitor after being administered for 50 days (data not shown). U0126 had no effects at the concentration of 5 μM .

To find whether any other growth factor has a similar promotive effect on RPE transdifferentiation, a combination of FGF-2 and one of factors (EGF, IGF-1, PDGF, CNTF or insulin) was tested on cultures of isolated RPE sheets to see whether the two factors synergistically promote cell growth and/or neural differentiation of RPE cells. EGF, IGF-1, PDGF or insulin alone showed no promotive effect. The combination of FGF-2 and IGF-1 (or insulin) was very effective (Fig. 8), while none of EGF, PDGF and CNTF had any effect even when combined with FGF-2. It is of interest that IGF-1 (or insulin) alone had no effect but was effective only when administered concomitantly with FGF-2.

When cultured in the presence of both FGF-2 and IGF-1, the RPE sheet expanded in size compared to FGF-2 alone (Figs. 8A, B), and the number of cells labeled for BrdU after 30 days was approximately twice compared with FGF-2 alone (Fig. 8D). On day 35, in some areas, cell sheets overlapped with each other due to the high growth activity. The effect of combinatory administration on neural differentiation was examined for HPC-1 and acetylated tubulin, and the immunoreactive cell numbers on day 35 were much higher in the cultures containing both growth factors than in those with FGF-2 alone (Figs. 8C, E, F). The effect of SU5402 at 20 μM was tested on the isolated RPEs cultured in the presence of both FGF-2 and IGF-1 to examine to what extent the inhibitor

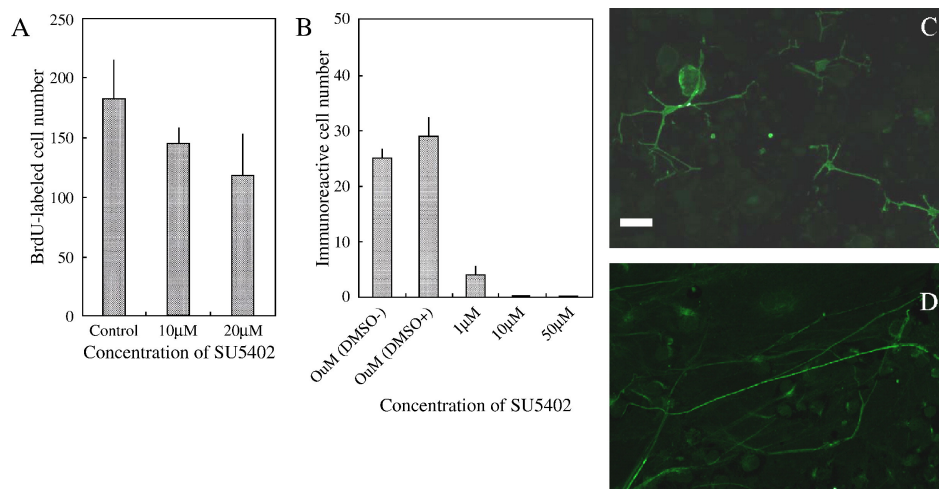


Fig. 6. Effect of SU5402 on the cell proliferation (A) and neural differentiation (B) in the RPE–choroid organotypic cultures. Differentiation of Syntaxin 1A-positive cells was mostly suppressed at 1 μM SU5402, while acetylated tubulin-immunoreactive cells appeared unaffected at 20 μM (C, D). Scale bar in panel C is 100 μm and is applied to panel D.

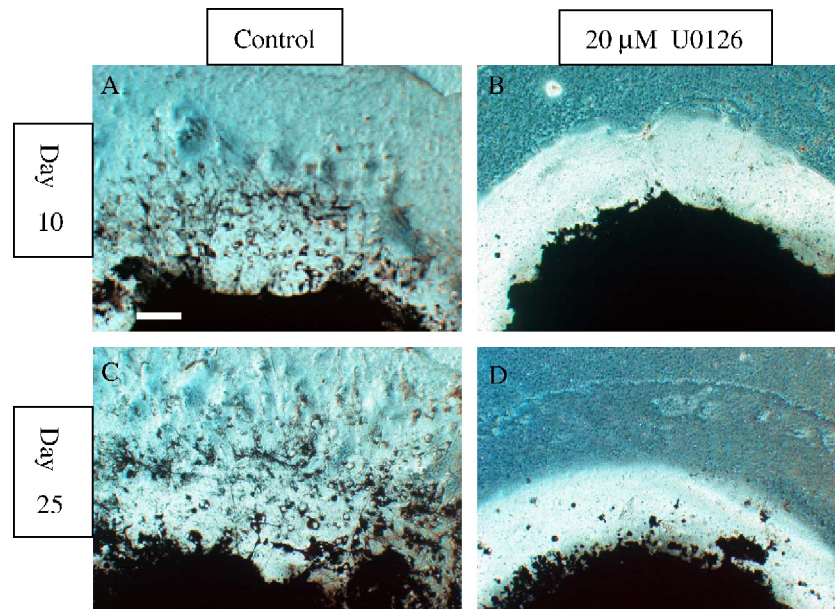


Fig. 7. Effect of U0126 on RPE transdifferentiation in the RPE–choroid organotypic cultures. In the presence of 20 μ M U0126, RPE cells did not migrate out of the explants. Cultures without U0126 (A, C) and with (B, D). Scale bar in panel A is 100 μ m and is applied to panels B, C and D.

influences the synergic effect on neural transdifferentiation. The result showed that the overall observations were fundamentally similar to those cultured without the inhibitor although neural differentiation appeared to be suppressed when examined by acetylated tubulin immunocytochemistry (Figs. 8C, E). In the presence of 20 μ M U0126, RPE cells did not undergo transdifferentiation even when cultured with FGF-2 and IGF-1 (data not shown), suggesting that FGF signaling pathway plays the central role in RPE transdifferentiation and that IGF-1 signaling may affect the pathway by enhancing the efficiency of signal transduction.

Detection of FGF-2 and IGF-1 gene expression in the RPE and choroid

We then examined whether FGF-2 and IGF-1 were actually produced in the choroids at least at the transcriptional level. Since the complete separation of RPE from the choroid was impossible within a short time, RPE and choroid were assayed altogether for RT-PCR analysis. Although relatively at a low level, gene expression of both FGF-2 and IGF-1 was detected in the RPE plus choroid. Interestingly, the expression level appeared to be up-regulated after surgical removal of the retina (Fig. 9).

Intraocular transplantation of cultured RPE sheets

To see whether cultured RPE has the potential to form a stratified retinal structure, cultured explants were grafted into the newt eye. RPE–choroid tissues cultured for 10 days and transplanted intraocularly formed a presumptive retinal layer clearly observed separating from the RPE layer ($n = 5$) (Figs. 10A, B). Although this retinal tissue did not show a matured

stratification, the observation indicates that the cultured RPE has the ability to form a layered structure if subjected to the appropriate conditions. In contrast, isolated RPE sheets cultured for 10 days in the presence of FGF-2 did not form multi-layered structures within the eye chamber in all cases examined ($n = 17$), and forming at most a three-layered epithelium, but without any organized stratification (Figs. 10C, D). Cells in the epithelial layer were often pigmented like RPE cells.

Discussion

A complete neural retina can be regenerated after surgical removal in adult animals only in certain urodele amphibians, but the cellular and molecular mechanisms involved are totally unknown, in contrast to the large volume of detailed descriptive knowledge (Hasegawa, 1958; Keefe, 1973; Levine and Cronly-Dyllon, 1974; Reyer, 1977; Stone, 1950a, b). Retinal regeneration in adult urodeles is achieved by two distinct pathways; one major source being the RPE cells that transdifferentiate into the retina and the other concerning the neural stem cells in the ciliary marginal zone. We focused on the transdifferentiation of RPE cells and addressed the question: what triggers RPE cells to transdifferentiate when the retina is lost? We previously described an *in vitro* culture model of newt tissue that allows RPE cells to transdifferentiate into neural cells (Ikegami et al., 2002). In the present study, we revealed that RPE cells need the choroid for their growth and neural differentiation but do not require the retina. This RPE–choroid interaction is mediated by diffusible factors, and FGF-2 and IGF-1 represent candidate molecules.

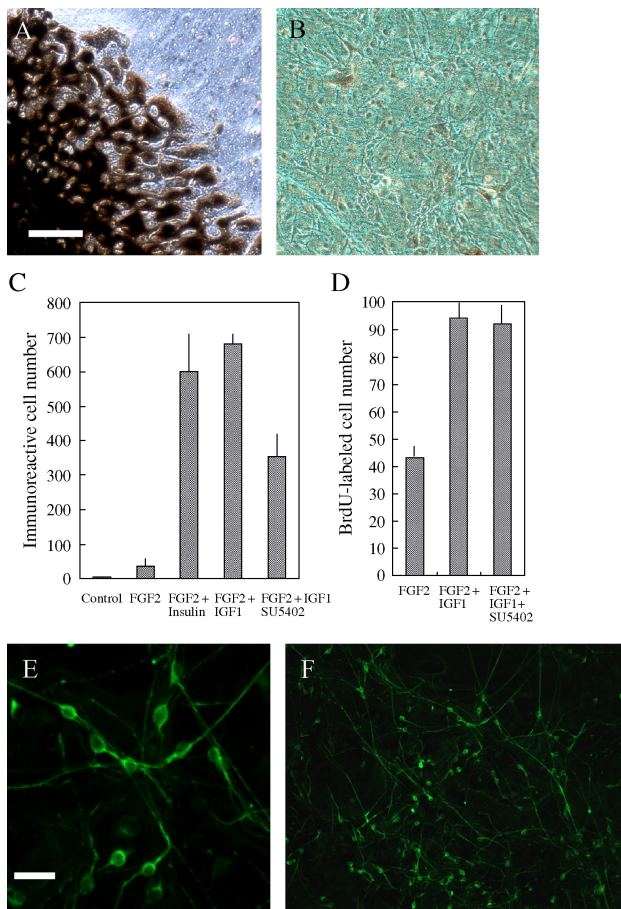


Fig. 8. Effect of a combined administration of FGF-2 and IGF-1 in cultures of isolated RPE sheets. (A) RPE cells cultured with IGF-1 showed an epithelial profile as seen in the normal culture in Fig. 2D, while RPE cells cultured with FGF-2 and IGF-1 rapidly underwent depigmentation and proliferation (B). (C, D) Both cell proliferation and differentiation were promoted in a combined administration of FGF-2 and IGF-1. SU5402 partially blocked neural differentiation but showed no effect on cell proliferation when administered in the presence of FGF-2 and IGF-1. Virtually no BrdU-labeled cells were found in RPE cultures without adding FGF-2. (E, F) Acetylated tubulin-immunoreactive cell numbers were increased drastically when cultured in the presence of both FGF-2 and IGF-1. Scale bar in panel A is 100 μ m and is applied to panel B. Bar in panel E is 100 μ m for panel E and 250 μ m for panel F.

RPE–choroid tissue interaction plays a key role in the initiation of retinal regeneration

A previous study showed that dissociated RPE cells from the newt eye proliferate and form an epithelial sheet under culture conditions. Some of these cells manifest Na^{2+} and Ca^{2+} ion channels although they are epithelial and do not show a neuronal morphology (Sakai and Saito, 1997). Initially, we had also attempted to use dissociated RPE cells and noticed that some cells proliferated and transformed to become depigmented flat cells that neither exhibited a neuronal shape nor any neuron-specific marker examined. Instead, we tried to obtain an RPE cell sheet without dissociating it to single cells to retain their intercellular communication intact. This is especially important in

comparing results obtained in the experimental culture model with those found in *in vivo* regeneration studies. The present method allowed us to obtain a single pure RPE cell sheet from a whole eyecup. Disperse treatment does not destroy epithelial cell adhesion structures and appears to cleave fibronectin and type IV collagen (Stenn et al., 1989). We used the enzyme at a very low concentration (50 unit/ml), which we speculate yields minimum damage to RPE cells. Immediately after placing in culture, the isolated RPE sheet formed a compact uniform cell arrangement similar to that seen *in vivo*. When RPE sheets were reaposed to the choroid and placed in culture, RPE cells proliferated, migrated and differentiated into neural cells and rod photoreceptors exactly in the same way as they would if the sheet was not removed from the choroid.

Histological preparations of disperse-treated tissues showed that the RPE sheet was detached from the Bruch's membrane, which remained attached to the choroid. It is possible that the loss of the Bruch's membrane prevented RPE cells to grow and differentiate into neurons, since there is considerable evidence that extracellular matrix (ECM) elements influence the state of epithelial cell differentiation (Hiscott et al., 1999; Saika et al., 2002; Werb and Chin, 1998). Several studies reported that some components of the basement membrane stimulate RPE cells of the *Xenopus* tadpole to transdifferentiate into retinal cells (Reh and Nagy, 1987; Sologub, 1977), but it was also shown that the RPE cells must detach from Bruch's membrane if they are to undergo the transition to neuroblasts (Lopashov and Sologub, 1972). Our preliminary results showed that ECM components such as fibronectin and laminin had no effects on RPE cell proliferation when used as a substratum for RPE cultures (data not shown). Alternatively, the choroid might affect RPE cells by diffusible substances that are secreted by certain resident cells. This possibility was examined with the ryomen culture method and the results showed that the choroid stimulated RPE cells to migrate and proliferate, although neuronal differentiation was not as intensive as seen in the combined RPE–choroid preparations. This might be partly due to the thickness of the filter membrane that separated RPE cells from the choroid. These results suggest that the tissue interaction between the RPE and choroid is mediated by some diffusible factors, although this does not necessarily completely rule out a role for the Bruch's membrane.

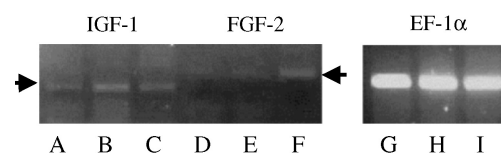


Fig. 9. Detection of FGF-2 and IGF-1 gene expression in the RPE and choroid by RT-PCR. Lanes A, D and G are from uninjured newts and lanes B, E and H and lanes C, F and I are from retinectomized newts at day 10 and day 15 post-operation, respectively. (A, B, C) IGF-1, (D, E, F) FGF-2, (G, H, I) EF-1 α as a control. More intense bands for IGF-1 and FGF-2 gene expression are detected in the retinectomized newts.

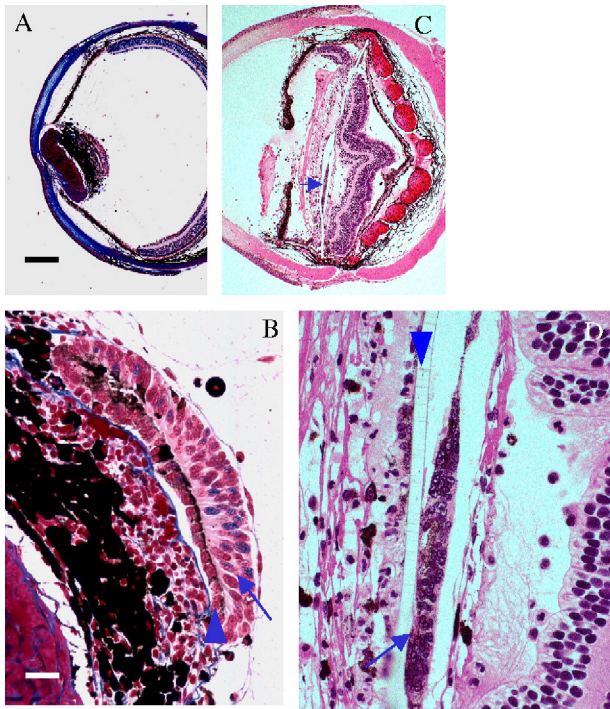


Fig. 10. Intraocular transplantation of organ cultured RPE–choroid (A, B) and isolated RPE (C, D). In panel B, two layers already separated, one appeared differentiating retina (arrow) and the other an RPE layer (arrowhead). In panel D, the graft (arrow) is located close to the host retina and is observed as a 2- or 3-cell layer but without any organized structure. Most of the cells were pigmented. No retinal differentiation could be seen. The arrowhead indicates a filter membrane on which RPE sheet was cultured before being transplanted. Panels A and B were stained with Azan and panels C and D with hematoxylin and eosin. Scale bar in panel A is 500 μ m and is applied to panel C. Bar in panel B is 100 μ m and is applied to panel D.

Effects of growth factors and other diffusible factors on newt RPE transdifferentiation

Several growth factors have been found to play a key role in retinal regeneration from RPE cells as studied *in vivo* (Park and Hollenberg, 1989) and *in vitro* (Araki et al., 1998, 2002; Pittack et al., 1991; Zhao et al., 1995). RPE cells from *Xenopus* tadpoles also differentiate to neurons *in vitro* under the effect of FGF-2 (Sakaguchi et al., 1997). To our knowledge, no studies have been performed on the molecular mechanism involved in the retinal regeneration of urodele species. The present organ–culture system is an excellent tool for the analysis of factors involved in newt retinal regeneration, because RPE cells initiate cell proliferation and neuronal differentiation in a similar time course and to a similar extent as observed in the *in vivo* regenerating eye (Ikegami et al., 2002).

By obtaining a large intact RPE monolayer sheet and culturing it alone, we were able to examine the direct effects of various cell growth factors on RPE transdifferentiation. FGF-2 initiated RPE cell proliferation but all other factors (EGF, IGF-1, IGF-2, PDGF and CNTF) examined were totally ineffective, both on growth and cell differentiation, although they are widely known as mitogens both *in vivo*

and *in vitro*. Since the results of the *ryomen* culture indicated involvement of some diffusible factor(s) between the RPE and choroid, we inhibited FGF signaling by applying potent specific pharmacological inhibitors (SU5402 and U0126) in the organ-cultured tissues of the RPE and choroid. The results indicated that FGF signaling pathway plays a central role in newt RPE transdifferentiation and suggest that a common mechanism regulates retinal regeneration in different vertebrate species, although retinal regeneration can be seen in the adult animal only in certain urodele amphibians. In the attempt to test whether any other growth factor has a similar effect like FGF-2, we found that IGF-1 greatly increased the effect of FGF-2, although sole administration of IGF-1 was totally ineffective for RPE transdifferentiation. RT-PCR survey revealed that the choroid synthesizes both FGF-2 and IGF-1 mRNAs in the newt eye, suggesting that these two growth factors act synergistically on RPE cells. It is not yet known whether these two factors exert different roles on RPE transdifferentiation, but the finding that FGF-2 was not effective on RPE cells when it was administered later than day 10 may suggest that FGF-2 allows RPE cells to re-enter cell cycle progression, while that IGF-1 enhances neural differentiation. Our previous observation that the initial uptake of BrdU begins about days 5–6 while neural differentiation starts about day 15 supports this notion. In addition, it has been reported that IGF-1 enhances newt lens regeneration (Connelly and Green, 1987).

Since SU5402 binds specifically to the active sites of FGF receptor 1 kinase domains (Mohammadi et al., 1998), FGF-2 is considered to exert its effects by activating not only FGFR1 but also other types of FGF receptors (FGFR2–4), although there is no information at the moment about which types of FGFRs the newt RPE cells possess and are involved in transdifferentiation. In the transgenic mice expressing dominant-negative FGFR1 in the pigmented cells, eye growth is strongly impaired during early embryogenesis (Rousseau et al., 2000), suggesting that FGFR1 expression in the RPE cells plays a major role in mouse eye development. On the other hand, FGF9 appears to play an important role in defining the boundary between the RPE and neural retina during the early development of the mouse optic vesicle (Zhao et al., 2001), and FGF9 is the preferred ligand for FGFR3. A more detailed experiment is needed to identify the molecules involved.

Similar effects by FGF-2 and insulin have been reported in newly hatched chick eyes, in which the combination of insulin and FGF2 stimulated Müller glia to dedifferentiate, proliferate and generate new neurons (Fischer et al., 2002), implying that exogenous growth factors might be used to stimulate endogenous glial cells to regenerate neurons in the central nerve tissue. It must be clarified further whether IGF-1 and FGF-2 are actually working as endogenous signaling molecules between the RPE and choroid during *in vivo* retinal regeneration of the newt.

A recent report by Imokawa and Brookes (2003) provided an interesting finding on newt lens regeneration that thrombin activation in the dorsal iris triggers the process in a similar fashion to that of muscle regeneration in the limb (Tanaka et al., 1999). It is possible that a similar mechanism underlies retinal regeneration, because the choroid is a capillary-rich tissue and hence is a potent source for cellular clotting factors. Although such molecules would presumably be lacking in our culture system, the present tissue culture model is highly suitable to assess such a possibility.

Retinogenesis of the cultured RPE cells

Intraocular transplantation of RPE tissues has been performed in amphibian species to elucidate the extrinsic cues or tissue interaction for RPE transdifferentiation (Bosco, 1988; Lopashov and Sologub, 1972; Mitashov, 1997). Isolated RPEs combined with other different tissues are usually grafted into either the anterior chamber or vitreous chamber of host eyes. Intraocular grafting is suitable to examine the three-dimensional morphogenesis of the graft. We transplanted tissue-cultured RPEs into the eye chamber to examine whether RPE cells can form a layered retinal structure. Most RPE–choroid tissues cultured for 10 days in vitro could differentiate into both RPE and retinal layers within the vitreous chamber of the host newt eye reminiscent of regenerating RPE. This indicates that cultured RPE cells acquire the capacity to organize into the RPE and retinal structures. In contrast, isolated RPEs lacking the choroid never regenerated a retinal structure, instead remaining a pigmented epithelial form. It was reported that RPEs of adult *Xenopus laevis* without Bruch's membrane could regenerate retina in the tadpole eye chamber (Sologub, 1977). It may be possible that isolated RPEs have lost the ability to respond to environmental signals from the host eye retina after maintained in vitro for a certain period, as was suggested by the observation that FGF-2 was not effective on RPE cells when it was administered later than day 10.

The present study clarifies the importance of the neighboring tissue underlying the RPE as a trigger of RPE cell transdifferentiation and implies the crucial role of the choroid. It also showed that retina-derived factor is not a necessary cue for RPE transdifferentiation. An important remaining question is how RPE or choroidal cells are able to recognize the removal of neural retina. It may be that the neural retina exerts inhibitory actions against the RPE cells and/or choroid by modifying communication through intercellular signaling molecules and their receptors. The authors are now investigating this hypothesis.

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